Capsid protein of cowpea chlorotic mottle virus is a determinant for vector transmission by a beetle

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Cowpea chlorotic mottle virus (CCMV) is a bromovirus transmitted by species of chrysomelid beetles, including the spotted cucumber beetle, Diabrotica undecimpunctata howardii Barber. An experimental system was set up to identify the viral determinant(s) of the beetle transmission of CCMV. Nicotiana clevelandii was selected as an experimental plant host because it supports the replication and accumulation of both CCMV and a second member of the family Bromoviridae, cucumber mosaic virus (CMV). Using a reverse genetic system for CMV, a cDNA copy of the CCMV capsid protein (CP) gene was substituted for that of the CMV CP gene. The resulting ‘CMV-hybrid’ consisted of wild-type CMV RNA1, RNA2, and a chimeric CMV RNA3 expressing the CCMV structural protein. The CMV-hybrid replicated and formed virions in N. clevelandii; in electron micrographs the hybrid virus was indistinguishable from CCMV. In beetle feeding assays, both CCMV and the CMV-hybrid were transmitted by D. undecimpunctata, while beetle transmission of CMV was not observed. Conversely, only CMV was observed to be transmitted by the aphid Myzus persicae. Surprisingly, the CMV-hybrid was transmitted more efficiently than the parental CCMV, and a virus-induced alteration in beetle feeding behaviour is proposed to account for the difference. These results indicate that the CCMV CP is a viral determinant for beetle vector transmission.

INTRODUCTION

Plant virus survival is, in most cases, intimately linked to associations with vectors. Arthropods, nematodes, fungi and protists can all function as vectors. The arthropods are numerically and economically the most important group of vectors. While hemipterans (suborder Sternorrhyncha) are the most common vectors (e.g. aphids and whiteflies), members of the order Coleoptera (beetles) are numerically significant. There are 61 beetle species that have been shown to transmit plant viruses, and plant virus transmission by beetles is relatively specific since other common plant virus vectors such as aphids and leafhoppers are not reported as vectors of beetle-transmitted viruses (Nault, 1997). Our knowledge of the beetle transmission of plant viruses is relatively limited, with the studies of Fulton, Scott, Gergerich and colleagues providing the bulk of the data (Fulton & Scott, 1980; Fulton et al., 1987; Gergerich, 2002; and references therein). Beetles can acquire and transmit virus without a latent period, harbouring virus and remaining viruliferous for many days, but the mode of transmission is not entirely clear. Mechanical feeding damage and regurgitation are associated with transmission (Freitag, 1956), and a tolerance to inhibitors of transmission in the regurgitant appears to be a requirement for beetle-transmitted viruses (Gergerich et al., 1983, 1991; Nault, 1997). An unusual property of beetle-transmitted viruses is their ability to be transported away from the wound site via the xylem and to establish an infection in unwounded plant cells distal from the feeding site (Gergerich & Scott, 1988). Virions of many beetle-transmitted viruses can be recovered from the haemolymph of vectors (Slack & Fulton, 1971), but its relationship to the process of transmission has not been clearly established.

There are 42 species of beetle-transmitted plant viruses, representing six genera: Bromovirus, Carmovirus, Comovirus, Machlomovirus, Sobemovirus and Tymovirus. These are all RNA plant viruses with two common properties: (i) they have similar virion morphologies with icosahedral symmetry and (ii) they exhibit a relatively high level of physical stability. Furthermore, most of these viruses and their beetle vectors have narrow host ranges (Fulton et al., 1987; Nault, 1997). Although the beetle-associated bromoviruses are relatively inefficiently transmitted by vectors (Lane, 1979), some such as cowpea chlorotic mottle virus (CCMV) are useful for experimental studies. CCMV is easy to genetically manipulate, with a well-developed reverse genetics system for functional
studies of viral-encoded proteins. CCMV is also transmitted by the spotted cucumber beetle, a vector that can be reared in the laboratory (Hobbs & Fulton, 1979; Walters & Dodd, 1969).

The goal of this study was to determine if the capsid protein (CP) of a beetle-transmitted virus was a determinant of vector transmission. This is the case for all non-enveloped, vector-transmitted plant viruses studied, with a range of vectors including arthropods (Gray & Bannerjee, 1999; Nault, 1997), fungi (McLean et al., 1994), protists (Tamada & Kusume, 1991) and nematodes (MacFarlane, 2003). Feeding studies using beetles fed upon purified virus have suggested that additional viral or host factors are not required (Gergerich, 2002; Langham et al., 1990). In this study, the expression of a CP from a beetle-transmitted virus conferred beetle transmissibility, and an unexpected virus-associated change in the feeding behaviour of the insect vectors was observed.

RESULTS

Construction of a hybrid virus

In order to evaluate the contribution of the CCMV CP to beetle transmission, we constructed a chimeric cDNA clone of CMV RNA3 expressing the CCMV CP. To facilitate this work, pFny309 was engineered with SpeI and EcoRV restriction enzyme sites flanking the 5′- and 3′-ends of the CP gene to create pFny309-Spe/Eco. An additional modification of the 3a movement protein (MP) was made to enhance the virus cell-to-cell movement potential. A stop codon was introduced near the 3′-end of the CMV 3a MP gene to truncate the C terminus of the MP gene by 33 aa; the resulting construct was designated pFny309-MPSM (movement protein stop mutant; Fig. 1). This mutation had previously been shown to potentiate the cell-to-cell movement of CP-less and chimeric viruses (Kim et al., 2004; Nagano et al., 1997, 2001). The CCMV CP gene was amplified with oligonucleotide primers containing SpeI and EcoRV restriction enzyme sites, and the fragment inserted into pFny309-MPSM to create pFny309-CCMV. The resulting chimeric virus derived from this infectious clone is referred to as the CMV-hybrid virus.

CMV-hybrid virus forms virions and induces a strong symptom phenotype

Transcripts of pFny109 (RNA1), pFny209 (RNA2) and pFny309-CCMV mechanically inoculated onto Nicotiana clevelandii gave rise to leaves with a mottle-mosaic, dwarfing and growth distortion 7–14 days post-inoculation. The appearance was akin to those induced by transcripts of pFny309-MPSM with the wild-type CMV CP gene, except the plants were not as severely dwarfed, and the symptoms included a bronzing and the induction of small (<1 mm) necrotic lesions.

Virions of the CMV-hybrid and CCMV were purified using a protocol developed for bromovirus isolation (Bujarski, 1998), while those of CMV-MPSM were purified with a standard CMV procedure (Lot et al., 1972); two purification procedures were required because the CPs have different physical properties and pH stabilities. RNAs were purified from virions, electrophoresed on a native agarose gel, and the relative migrations were compared (Fig. 2). RNAs 3 and 4 of the CMV-hybrid migrated to positions different from the parental CMV-MPSM and CCMV.

![Fig. 1. Schematic of the modified CMV RNA3 cDNA in the plasmid pFny309-MPSM. Infectious transcripts from pFny309-MPSM gave rise to the virus CMV-MPSM. The insert depicted shows the encoded RNA3, 3a MP and CP gene. The positions of the 5′- and 3′-ends of the encoded RNA are indicated. The larger arrows indicate the positions of the stop codons for the wild-type 3a MP gene and the mutated gene in the parental pFny309-MPSM. The introduced stop codon gives rise to a truncation of the C-terminal 33 aa of the 3a MP. The smaller arrows show the positions of the engineered SpeI and EcoRV sites that were used to excise the CMV CP gene and replace it with the CCMV CP gene.](image1)

![Fig. 2. Agarose gel electrophoretic RNA profiles of CMV, CMV-hybrid and CCMV. RNAs purified from virions (2 µg) were electrophoresed on a 1% native agarose gel. Lanes 1, CMV-MPSM; 2, CMV-hybrid; 3, CCMV. The positions of the CMV-MPSM and CCMV RNAs are indicated to the left and right hand sides of the figure, respectively.](image2)
RNAs. Although this was a native gel and RNA migration cannot be directly related to size, the RNAs 4 of the hybrid virus could clearly be seen as migrating differently from the two parental viruses. This was consistent with the smaller size of the RNA4-encoded CP gene of CMV compared with that of CMV (573 versus 657 nt). The full-length RNA3 of CCMV is also shorter than that of CMV-MPSM [2173 (Allison et al., 1989) versus 2216 nt (Owen et al., 1990)]. The predicted sizes of the RNAs 4 of CMV-MPSM, CMV-hybrid and CCMV are 1034, 950 and 824 nt, respectively. The amounts of RNAs 3 and 4 relative to RNAs 1 and 2 appeared lower in the CMV-hybrid versus the parental viruses. While this was not investigated further, there was no apparent impact on the accumulation of CP in CCMV versus CMV-hybrid (discussed below).

When viewed by transmission electron microscopy, the CMV-hybrid virions resembled those of CCMV, the virus from which the CP gene was derived. While the overall appearance of all virions was similar at this resolution, those of CCMV and the CMV-hybrid appeared slightly smaller than those of CMV-MPSM. In a limited number of measurements \(n=20\); virions as shown in Fig. 3), the mean CMV-hybrid virion diameter (29.0 nm) was 12% less than the mean CMV-MPSM diameter (32.8 nm). This size difference is consistent with early descriptions of CMV and CCMV viewed by transmission electron microscopy (Bancroft, 1971; Francki et al., 1979) and with virion size estimates from crystallographic studies of 284 Å for CCMV (native) and 305 Å for CMV (Smith et al., 2000; Speir et al., 1995).

**CCMV and the CMV-hybrid are beetle-transmitted, but not aphid transmissible**

To assess whether CCMV and the CMV-hybrid were beetle transmissible, transmission experiments were carried out using spotted cucumber beetles and *N. clevelandii*. In a preliminary set of transmission experiments comparing CCMV with the CMV-hybrid, transmission of CCMV was observed in 4 of 45 plants and the CMV-hybrid was transmitted to 8 of 44 plants (one plant died); no transmissions were observed with beetles fed upon uninfected plants. This confirmed that the parental CCMV was beetle transmissible, and showed that the CMV-hybrid virus with the CCMV CP was also transmitted by the spotted cucumber beetle. A second set of transmission experiments with CMV-MPSM controls were performed and the data are shown in Table 1. In each of six replicated experiments, the CMV-hybrid was transmitted to a minimum of three of eight plants. While transmission of CCMV was observed in half of the experiments, the beetle transmission of CMV-MPSM was not observed. These results demonstrate that the beetle transmission phenotype is conferred by the CCMV CP.

A reciprocal set of experiments was performed in order to test for the aphid transmissibility of the viruses. Aphids are not reported as specific vectors for members of the bromovirus group (Lane, 1979). In four independent experiments, CMV-MPSM was efficiently transmitted by *Myzus persicae* to *N. clevelandii*; all 20 plants exposed to aphids were infected (20/20), with symptoms appearing 5–7 days after plant inoculation. No aphid transmissions were observed for CCMV, the CMV-hybrid or from virus-free plants with ratings of 0/20 plants infected in all cases. In each of the four experiments, there were five target plants and 50 aphids used per virus (i.e. a total of 200 aphids for each virus).

**Feeding behaviour as a mechanism to account for the lower beetle transmission efficiency of CCMV**

An unexpected observation in beetle transmission experiments was that the parental CCMV was transmitted at a lower frequency than the CMV-hybrid. In the initial set of experiments, the likelihood of CCMV transmission by a single beetle \(P^*\) was approximately half that of the CMV-hybrid; \(P^*=0.018\) (for 4/45 plants infected) versus \(P^*=0.039\) (for 8/44 plants infected), respectively. This same pattern was observed in the six replicated experiments where CCMV was transmitted at an equal or lesser frequency than the CMV-hybrid (Table 1). The mean estimated likelihood of transmission by single beetles for CCMV was \(P<0.038\), an estimate that differed significantly from the mean for the CMV-hybrid of \(P^*=0.162\) \((P<0.01)\). The \(P^*\) estimate of 0.038 for CCMV is an overestimate of the true value, as a single transmission event was assumed for the three experiments where no transmission was observed (discussed in Methods).

During the course of the experiments, beetle feeding on CCMV-infected leaves appeared to be less than feeding on the CMV-hybrid-infected leaves. This was postulated to be the reason for the lesser transmission. To quantify feeding,
Table 1. Transmission of the viruses CCMV, CMV-MPSM and the CMV-hybrid to N. clevelandii by the spotted cucumber beetle, D. undecimpunctata

Transmission efficiency is expressed as the number of plants infected/number of plants exposed to beetles. The number in parentheses is \( P^* \), the estimated likelihood of transmission by a single beetle; that for the total is the mean value of \( P^* \) (see text for methods).

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Virus</th>
<th>CCMV</th>
<th>CMV-MPSM</th>
<th>CMV-hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/6 (0.036)*</td>
<td>0/6</td>
<td>5/8 (0.178)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3/8 (0.090)</td>
<td>0/8</td>
<td>3/8 (0.090)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0/8</td>
<td>0/8</td>
<td>6/8 (0.242)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0/8</td>
<td>0/8</td>
<td>3/8 (0.090)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1/8 (0.026)</td>
<td>0/8</td>
<td>6/8 (0.242)</td>
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</tr>
<tr>
<td>6</td>
<td>0/8</td>
<td>0/8</td>
<td>4/8 (0.129)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5/46 (&lt;0.038)</td>
<td>0/46</td>
<td>27/48 (0.162)</td>
<td></td>
</tr>
</tbody>
</table>

*The experimental unit was five beetles per plant and eight plants per treatment (except experiment 1).

Fig. 4. Beetle feeding on detached leaves of N. clevelandii virus-free or infected with CCMV, CMV-MPSM or CMV-hybrid virus. Data presented are combined from two experiments with six replications per treatment in each experiment. Bars with different letters are statistically different at \( P=0.05 \).

Using a hybrid virus system based on CMV, the functional expression of the heterologous CP of the beetle-transmitted CCMV conferred beetle transmissibility. In the CMV-hybrid virus, an altered cucumovirus MP and a bromovirus CP functioned cooperatively to facilitate both the cell-to-cell and systemic movement in the common host, N. clevelandii. Wild-type CMV and CCMV both require a CP for cell-to-cell movement. The cDNA construct encoding these proteins (pFny309-CCMV) was based on the RNA3 of CMV-Fny, but with two significant changes. In the first change, the MP gene was modified to express a truncated protein without the C-terminal 33 aa. This change was introduced because it allows for the CP-independent cell-to-cell movement of CMV (Kim et al., 2004; Nagano et al., 1997). The strategy was adopted, because in some studies among members of the family Bromoviridae, the cognate CP is required for wild-type MP-mediated cell-to-cell movement (Nagano et al., 1999). Furthermore, even for exchanges of the wild-type MPs or CPs among members of the genus Cucumovirus (e.g. CMV and TAV) the progeny are not all viable and able to systemically infect a common host (Salanki et al., 1997, 2004). The complexity of such interactions is highlighted by the fact that viability can be host dependent. While the CMV-hybrid was able to infect N. clevelandii, a common host for both viruses, it did not systemically infect Nicotiana tabacum cv xanthi NN, a host for CMV, but non-host for CCMV (A. J. Clark & K. L. Perry, unpublished).

Two additional observations from this study are significant. Firstly, the CCMV CP appeared to specifically encapsidate CMV RNAs (Fig. 2). This contrasts with a previous study using a different bromovirus, in which the coat protein gene from brome mosaic virus (BMV) was unable to confer encapsidation of the heterologous CMV RNAs (Osman et al., 1998). In that study, a chimeric CMV expressing the BMV CP (analogous to the CMV-hybrid in this study) was inoculated into N. benthamiana protoplasts; the virus replicated and BMV CP was confirmed as being expressed by Western analysis, but virions and virion encapsidated CMV RNAs could not be detected by Northern analysis. By contrast, the reciprocal chimera of BMV expressing the CMV CP, was shown to produce virions, and encapsidated BMV RNAs were observed (Osman et al., 1998). Secondly, beetle feeding behaviour was altered on plants infected with CMV-Fny and the
CMV-hybrid relative to plants infected with CCMV or uninfected plants. This bias in feeding behaviour was observed throughout the course of six transmission experiments, and subsequently quantified in two feeding experiments (Fig. 4). The differential feeding behaviour was postulated to account for the rather surprising result that the beetle transmission efficiency of the parental CCMV was less than that of the CMV-hybrid in all but one experiment (equal in the remaining experiment; Table 1). We cannot rule out the possibility that differences in the concentrations of virions in CMV-hybrid- versus CCMV-infected plants may have influenced transmission, but this was not suggested by results from ELISA assays of source leaves. Virus infection of plants is known to affect herbivore–plant interactions, but most effects are specific to the system of study and are not generalized (reviewed by Fereres & Moreno, 2009). For instance, a potyvirus infection of soybean plants reduced the residence time of *Rhopalosiphum maidis* but not that of a second aphid, *M. persicae* (Fereres et al., 1999). There are multiple examples of vectors benefitting from feeding on virus-infected plants, but the effects are usually longer-term, affecting fecundity or migration potential (Fereres & Moreno, 2009). By contrast, the effects observed in this study appear to be that of a virus enhancing vector feeding behaviour, perhaps by suppressing a host defence response.

In previous studies, CCMV isolates were transmitted by at least four species of beetles, including the bean leaf beetle *Ceratoma trifurcata* and the spotted cucumber beetle *Diabrotica undecimpunctata howardii* Barber (Gamez, 1972; Hobbs & Fulton, 1979). CCMV and other members of the genus *Bromovirus* are described as less efficiently beetle-transmitted than other beetle-vectored viruses (Hobbs & Fulton, 1979; Monis et al., 1986). Although bromovirus beetle transmission efficiency may be low, the specificity with regards to the beetle vector is still apparent. CCMV is beetle vectored, but not (or rarely) transmitted by aphids, as observed in this study. The converse relationship applies to CMV; although this virus is aphid-vectored, no beetle transmission was observed in this study or has previously been reported.

The results of this study provide experimental evidence to show that the CCMV CP is a sufficient determinant of beetle vector transmission. Of perhaps broader interest is the observation that the CMV-hybrid was transmitted more efficiently than the parental CCMV. It will be of interest to identify the genetic basis in these viruses for this difference in vector transmissibility.

**METHODS**

**Virus maintenance, propagation, purification and electron microscopy.** Virus isolates CMV-Fny, CCMV-T and their RNA3 sequences have been described previously (Allison et al., 1989; Owen et al., 1990) and are from the virus collection at Cornell University. Viruses were propagated in *N. clevelandii*, grown under greenhouse conditions, and purified by standard methods (Bujarski, 1998; Lot et al., 1972). Purified virus was stored at −20 °C in the respective resuspension buffers for CCMV (Bujarski, 1998) and CMV (Lot et al., 1972) with 50% glycerol. Plants for vector transmission experiments were mechanically inoculated with aliquots of purified virus diluted in storage buffer at concentrations from 0.7 to 40 μg ml⁻¹.

The infection status of plants was assessed by monitoring visual symptoms and by ELISA (Clark & Adams, 1977; Mowat & Dawson, 1987) using polyclonal antisera (He et al., 1998). To facilitate comparisons between plants and experiments, ELISAs samples were prepared from 0.1 g tissue pulverized in 1.0 ml sample extraction buffer (0.015 M sodium carbonate and 0.035 M sodium bicarbonate, pH 9.6), resulting in a semiquantitative assay (Mowat & Dawson, 1987).

Virions were visualized using a FEI Philips TECNAI 12 BioTwin transmission electron microscope (FEI Corporation) following staining with 3% uranyl acetate; size estimates were made using an internally calibrated standard for the instrument.

**Engineering restriction sites flanking the CP gene.** PCR-based site-directed mutagenesis was used to introduce restriction sites on either side of the CP gene in a cDNA clone of CMV-Fny RNA3, pFny309 (Rizzo & Palukaitis, 1990). The strategy for the generation of mutated PCR fragments was based on the procedure of Higuchi et al. (1988), as described previously. The high fidelity, thermostable polymerase *Pfu* (Stratagene) was used according to the manufacturer’s instructions with the following amplification conditions: 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 5 min. Recombinant DNA techniques were performed using standard procedures (Sambrook et al., 1989). An *Sphl* site (underlined) was introduced upstream of the CMV CP gene start site (in bold; coding sequences italicized) using an oligonucleotide primer with the sequence 5′-CGTACATCTTGATATCGGGCAAATC-3′, corresponding to positions 1238–1267 in CMV RNA3 (GenBank accession no. D10538; Owen et al., 1990) except for the introduced mutations at nt 1250–1255. The *Sphl* primer was used in combination with a 3′-end primer with the sequence 5′-CTATGGCTCGTGTGCTC-CTCCT-3′ corresponding to the terminal 9 nt of RNA3 (in italics; positions 2216–2208) and including an introduced *Pstl* site (underlined). In order to produce the second PCR-mutated fragment required for the Higuchi procedure, a primer complementary to the initial mutagenic primer was synthesized and used in combination with a 5′-end primer 5′-CTGAAAGATATGCGGTTGCTGTGTTCCCAGAAATC3′ corresponding to CMV RNA3 nt 1–15 (italics) and containing a *BamHl* site (underlined). An *EcoRl* site was introduced downstream of the CMV CP gene (coding sequences italicized) using a primer with the sequence 5′-CTGCTCCAGTC- TGATATCGTAGTTCCAGAATC-3′, corresponding to CMV RNA3 nt 1901–1931 except for the two modified nucleotides (underlined); this primer and its complement were used as described above. The resulting cDNA clone was designated pFny309-Spe/Eco.

**Mutagenesis of MP.** To produce a hybrid CMV clone with an altered capacity for cell-to-cell movement, a stop codon was introduced downstream of the CMV CP gene (coding sequences italicized) using a primer with the sequence 5′-CGTACATCTTGATATCGGGCAAATC-3′, corresponding to CMV RNA3 nt 849–867. The single introduced nucleotide change (underlined) created a stop codon (in bold) that truncates the C terminus of the MP by 33 aa. The template used was pFny309-Spe/Eco and the clone produced was designated pFny309-MPSM. Virus obtained using transcripts derived from this plasmid are referred to as CMV-MPSM.

**Substitution of CCMV CP, in vitro transcription of viral RNAs and the inoculation of transcripts.** The wild-type CP gene of CCMV (Allison et al., 1989) was amplified, as described above, using PCR primers 5′-GGAAATTGATAGTTATGCTGCTAGCATGCTGTTCCAGAATC-3′ and 5′-GGGAACGCTCTTCAGCGGTTAGTGTTCCCAGAAATC-3′, producing a 0.7 kbp fragment. A hybrid CP was then constructed with the introduction of a *SpeI* site (underlined) using the PCR fragment and an *EcoRl* site (underlined) using an *EcoRl* fragment amplified from the cDNA clone of the CCMV CP (Owen et al., 1990). A 1.2 kbp fragment was then generated by *SpeI* digestion of the resulting hybrid CP plasmid, which was used in the production of an RNA3 fragment. A 1.2 kbp fragment was then generated by *SpeI* digestion of the resulting hybrid CP plasmid, which was used in the production of an RNA3 fragment. A 1.2 kbp fragment was then generated by *SpeI* digestion of the resulting hybrid CP plasmid, which was used in the production of an RNA3 fragment.
C CGGAGTG-3' corresponding to CCMV RNA3 nt 1337–1373 and 1917–1952; the underlined sequences correspond to the introduced SpeI and EcoRV sites, respectively. The purified product and the plasmid pFny309-MPSM were digested with SpeI and EcoRV, separated by agarose gel electrophoresis and purified. The CCMV CP containing the PCR product was ligated into the linearized plasmid vector. The insert in the resulting clone, pFny309-CCMV was completely sequenced prior to in vitro transcription.

Capped, in vitro transcripts from the full-length cDNA clones pFny109 (RNA1), pFny209 (RNA2) (Rizzo & Palukaitis, 1990) and clones based on pFny309-MPSM (RNA3) were synthesized using a MEGAscript T7 transcription kit (Ambion). Transcripts were mechanically inoculated onto carborundum-dusted leaves of *N. clevelandii* and virus was purified 14–21 days after inoculation.

**Beetle and aphid feeding and vector transmission.** Beetle transmission experiments were performed using teneral (newly hatched adult) spotted cucumber beetles (*D. undecimpunctata*), commercially purchased (French Agricultural Research, Lamberton, MN). Source plants infected with CMV, CCMV or the CMV-hybrid were prepared by mechanical inoculation 3–4 weeks prior to use. Unfed, newly emerged beetles were transferred to Petri dishes containing 2.0 g virus-infected leaves of *N. clevelandii* on a sheet of filter paper, with 10 beetles per plate. Plates were incubated under constant light at 25 °C for 24 h. Beetles were transferred to 4-week-old *N. clevelandii* plants, with five beetles onto each of eight plants. The plants with beetles were caged for 24 h, after which beetles were manually removed and plants transferred to a greenhouse. Three to four weeks after beetle exposure, plants were tested for virus by ELISA. Experiments were replicated six times. In a preliminary set of beetle transmission experiments, 45 paired comparisons of CCMV transmission versus CMV-hybrid transmission were made, but since variable numbers of plants were used on different days, the results are presented as a composite. To facilitate comparisons of transmission frequencies between experiments, estimates of the likelihood of transmission by single beetles were made using group-testing methods (Swallow, 1985). The maximum-likelihood estimator $P^*$ (Gibbs & Gower, 1960) was calculated as described previously (Ng & Perry, 1999; Perry & Francki, 1992). In experiments where no transmission was observed, a single beetle transmission event was assumed and the estimator $P^*$ thus represents an overestimate. This avoids the likely underestimating of the frequency of single transmission events inherent in group-testing methods (Gildow et al., 2008; Perry & Francki, 1992; Swallow, 1985).

The feeding behaviour of beetles was evaluated by placing five beetles in each Petri dish with 1.5 g leaf material. After 24 h, beetles and frass on the leaf surface were removed and leaves weighed. The experimental design was randomized; each treatment had six replicates (Petri dishes) and the experiment was performed twice. Data were evaluated using the PROC MIX on SAS software 9.1 (SAS Institute Inc.); replicates were considered random and experiments were considered a blocking factor in the statistical model.

The aphid transmission phenotype of the CMV-hybrid, CMV and CCMV was assessed using a local clone of the aphid *M. persicae*. Aphids were reared on turnip (*Brassica rapa var. rapa*) in growth chambers at 25 °C with a 16:8 h light:dark cycle. Apterae (non-winged forms) were collected and starved for 2 h prior to transmission experiments. The experimental unit was a single (virus-infected) source plant and five target plants, each with 10 aphids. For each replicate, aphids were fed on a *N. clevelandii* source plant infected with CCMV, CCMV, the CMV-hybrid or a no virus control and was observed for feeding behaviour with a virus acquisition access period of 0.5–5.0 min. Ten aphids were transferred onto each of five target plants. Following a 24 h inoculation access period, plants were sprayed with the insecticide acephate (Orthene; Valent USA Corporation) and transferred to a greenhouse. Transmission was evaluated by ELISA 3 weeks following transmission, and experiments were performed four times. Data for the maximum-likelihood estimator $P^*$ of two independent experiments were bulked and evaluated using the PROC MIX on SAS software 9.1 (SAS Institute Inc.); replicates were considered random and experiments were considered a blocking factor in the statistical model.

**ACKNOWLEDGEMENTS**

We thank Laura Miller for technical assistance, Dr M. Payton (Oklahoma State University) for statistical analyses and Dr Peter Palukaitis for helpful discussions. The helpful comments of two anonymous reviewers are also acknowledged.

**REFERENCES**


Beetle vector transmission


